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Short communication

Biolistic mediated site-specific integration in rice

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Abstract

Cre-*lox* mediated site-specific integration in tobacco or *Arabidopsis* used polyethylene glycol or *Agrobacterium*, respectively, to deliver the integrating DNA. The polyethylene glycol method is inconvenient since it requires the use of protoplasts. The *Agrobacterium* method is inefficient as the single-stranded T-DNA is not a substrate for Cre-*lox* recombination. In this study, we tested the biolistic method for the site-specific insertion of DNA into the rice genome. Two target callus lines, each harboring a single genomic *lox* target, were generated by *Agrobacterium*-mediated transformation. The target callus lines were subjected to a second round of transformation by particle bombardment with a construct designed to excise the plasmid backbone from the integrating DNA, followed by the recombination of the integrating DNA into the genomic *lox* target. Site-specific integration was obtained from both target callus lines. Three integrant plants were regenerated from one target line and were found to have a precise copy of the integrating DNA at the target site, although only one plant has the integrating DNA as the sole copy in the genome. Site-specific integration through the biolistic delivery of DNA can be considered for other plants that are transformable *via* particle bombardment.

Current plant transformation methods integrate DNA at random locations and often in a complex pattern of multiple intact and/or partial copies (Mabool and Chritou 1999). The lack of site-specificity means that chromosomal influences on transgene expression are likely to differ among independent transformants (Meyer 2000). Complex multicopy patterns add another concern as they are often linked to gene silencing (Iyer et al. 2000; Matzke et al. 2000; Muskens et al. 2000). In mice, single-copy site-specific insertions can be obtained through gene targeting *via* homologous recombination, but in higher plants, this approach is not yet practical (Puchta and Hohn 1996; Vergunst and Hooykaas 1999). On the other hand, gene targeting in tobacco (Albert et al. 1995; Day et al. 2000) and *Arabidopsis* (Vergunst et al. 1998; Vergunst and Hooykaas 1998) has been reported with recombinase-mediated site-specific integration.

With the recombinase-mediated approach, a recombination target is first inserted into the plant genome through random integration. Subsequent delivery of new DNA occurs at the genomic target catalyzed by the recombinase protein. Several site-specific recombination systems have properties suitable for directing DNA integration in plants (Ow and Medberry 1995). One such system from bacteriophage P1 is known as Cre-*lox*, where Cre is the 38.5 kD recombinase that recognizes 34 bp *lox* sites. In a recent report on Cre-*lox* mediated site-specific integration in tobacco Day et al. 2000), precise single-copy integration into genomic *lox* sites was found in nearly a third of the selected events. Within a given target line, reproducible transgene expression was obtained in over half of the precise single-copy integrants. Chromosome position effects were found, as different target sites conferred different expression levels on the introduced transgene. This implies that even though recom-

bination targets are introduced randomly, once a target site is deemed suitable for transgene expression, subsequent DNA delivery to the specified location would yield reproducible expression in a sizable fraction of precise insertions.

In an attempt to extend the range of crop plants that can be transformed *via* Cre-*lox*-directed integration, we chose to test a DNA insertion strategy in rice. In previous reports on Cre-*lox* DNA integration, the integrating DNA was delivered to tobacco protoplasts by polyethylene glycol-mediated transformation Albert et al. 1995; Day et al. 2000) or to *Arabidopsis via Agrobacterium* infection (Vergunst et al. 1998; Vergunst and Hooykaas 1998). The polyethylene-glycol method yields site-specific events at high efficiency, but the regeneration of plants from protoplasts is cumbersome for many crop plants and is not a routine procedure for rice. *Agrobacterium* mediated transformation is much more convenient, but the *Agrobacterium* transferred DNA is single-stranded, which is not a recombination substrate for Cre. Indeed, the efficiency of Cre-*lox* DNA integration in *Arabidopsis* was low. In this study, we tested the biolistic method for delivering the integrating DNA into rice calluses for Cre-*lox* site-specific integration.

Experimental design

The site-specific integration strategy is shown in Figure 1. To generate target cell lines, the fragment within pVS52, bracketed by the T-DNA borders, was introduced into rice cultivar T309 by *Agrobacterium* (super-virulent strain AGL1)-mediated transformation of scutellar callus. Plasmid pVS52 is a binary vector in a pPZP200 (Hajdukiewicz et al. 1994) backbone and contains a *cre* gene transcribed by a maize ubiquitin promoter (designated as *P*2). A *lox76* mutant sequence is imbedded into the untranslated region of the *cre* mRNA. Adjacent to *P2-lox76-cre* is a selection marker: a hygromycin phosphotransferase (*hpt*) coding region transcribed by the cauliflower mosaic virus 35S RNA promoter (*35S*). The cells transformed with pVS52 were selected in the presence of hygromycin (50 mg/l). From the analysis of six randomly selected callus lines, two lines, t5 and t6, were found to contain a single full-length copy of pVS52 (data not shown) and therefore were suitable target lines for site-specific integration of pVS55. With a pBluescript SK backbone, pVS55 contains a promoterless neomycin phosphotransferase coding region (*npt*) and

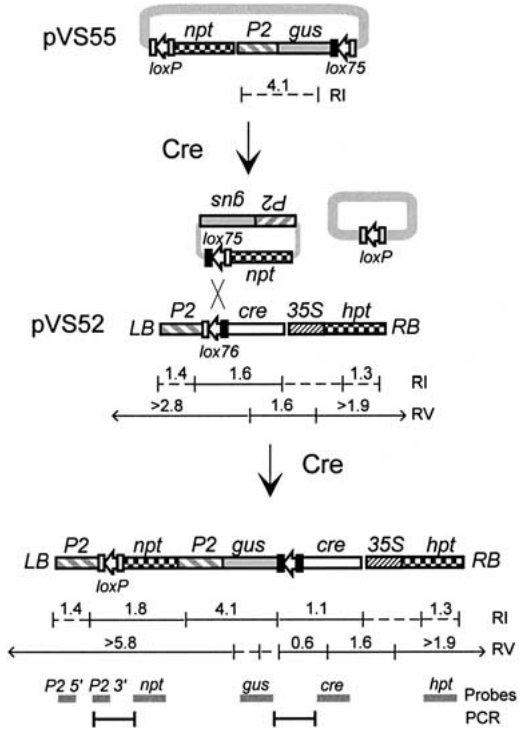

Figure 1. Site-specific integration in rice. Schematic (not to scale) representation of the integration construct pVS55 (top), the resolution of pVS55 into two separate molecules and the subsequent integration of one of the molecules into a genomic pVS52 target site (middle) to produce the resulting single copy integration structure (bottom). Only relevant features are shown. The 34 bp *lox* site is depicted as rectangles flanking an arrowhead to indicate, respectively, the 13 bp inverted repeat surrounding the asymmetric 8 bp core sequence. Mutant 13 bp sequences are indicated as closed rectangles (Albert et al. 1995). *Eco*RI and *Eco*RV sites and fragment sizes are shown. Dashed lines indicate hybridization fragments not presented in Figures 2. The extent of the hybridization probes and PCR products are shown at the bottom. Abbreviations used: *RB*, T-DNA right border; *LB*, T-DNA left border; RI, *Eco*RI; RV, *Eco*RV.

a β-glucuronidase coding region (*gus*) under the control of *P*2. This *npt-P2-gus* fragment is flanked by a *loxP* site upstream of *npt* and a *lox75* mutant site downstream of *gus*. The *loxP* and *lox75* sites can recombine with one another (Albert et al. 1995). Cre-mediated intra-molecular recombination between *loxP* and *lox*75 separates pVS55 into two molecules: a circular plasmid backbone with a *loxP* site, and a circular fragment consisting of *lox75-npt-P2-gus*. Insertion of the circular fragment into the genomic *lox*76 target forms a *P2-loxP-npt* linkage and thereby confers geniticin resistance. Moreover, *cre* transcription terminates through displacement of *cre* from its promoter, resulting in a *gus-lox75/76-cre* junction (Figure 1, bottom).

Table 1. Cre-*lox* mediated site-specific integration in rice.

Integrant lines	GUS activity	Number of clones	PCR			Southern	
			P2-npt	*gus-cre*	*P2-cre*	Site-specific	Random
t5 integrant calluses	+	11	11	11	11		
	−	11	11	9	11		
t6 integrant calluses	+	8	8	8	8		
	−	6	6	5	6		
t6-integrant plants	+	Integrant-1				1 copy	None
	+	Integrant-2				1 copy	∼2 copies
	+	Integrant-3				1 copy	>6 copies

Integrant calluses

Cultured cells of target lines t5 and t6 were bombarded with gold particles coated with pVS55 and selected on N6 media containing geneticin (100 mg/l). To avoid collecting sibling clones, only one geneticin-resistant callus was retrieved from each bombarded plate. Table 1 lists a total of 22 clones collected from t5, and 14 clones from t6. The presence of the two recombinant junctions (*P2-npt* and *gus-cre*) and the target junction (*P2-cre*) was assessed by PCR. The selected *P2-npt* junction was found in all 36 lines. The unselected *gus-cre* junction was also found in all but 3 clones. These 3 failed to express *gus*, but there were many other clones that lacked *gus* expression despite having both integration junctions (Table 1). For these insertions, the possibilities exist that the DNA between the two junctions either acquired a mutation, or was silenced. Since site-specific integration does not preclude the random integration of additional copies into the genome, and because random integrations generated by the biolistic method are generally complex structures, homology dependent gene silencing could be a contributing factor. Alternatively, or additionally, recent work in tobacco showed that even a precise single transgene copy at the target site could be silenced, through specific DNA imprinting of the newly introduced DNA (Day et al. 2000).

Surprisingly, a PCR product corresponding to the *P2-cre* target junction was detected in every callus line, which indicates that the geneticin-resistant clones were chimeric with target cells (without site-specifically integrated DNA) growing alongside the integrant cells. Due to this finding, we confined further molecular analysis to regenerated plants.

Integrant plants

For this biolistic-mediated integration system to be useful, regenerated plants must harbor the site-specific event. The integrant callus clones that showed *gus* expression were transferred to regeneration media. However, over a period of 4 months, shoot regeneration was successful only for one t5-derived and for four t6-derived plants. The t5-derived plant and one t6-derived plant were false positives that escaped selection and indeed hybridization analysis revealed the same transgene configuration as the corresponding t5 and t6 callus lines (data not shown).

The other three t6-derived plants, however, were found to harbor a site-specifically integrated transgene, with an integration structure consistent with that depicted in Figure 1. When *Eco*RI-cleaved DNA was hybridized to a *gus* probe (Figure 1), a 4.1 kb band was seen in integrant-1, but not in the t6-escape control (Figure 2a). When probed with *npt*, integrant-1 showed a 1.8 kb *P2-npt* band while t6 DNA failed to hybridize (Figure 2b). The 1.8 kb band was also detected by a *P2 3′* end segment (between *Eco*RI and *lox* sites), consistent with a *P2-lox-npt* linkage (Figure 2c). The *P2 3′* probe detected the expected 4.1 kb *P2-gus* fragment in integrant-1 and a 1.6 kb *P2-cre* band in t6. This 1.6 kb band was also found by the *cre* probe in t6, but was reduced to 1.1 kb in integrant-1 (Figure 2d). With *Eco*RV cleaved DNA, *cre* detected in t6 the expected 1.6 kb band and a >2.8 kb T-DNA

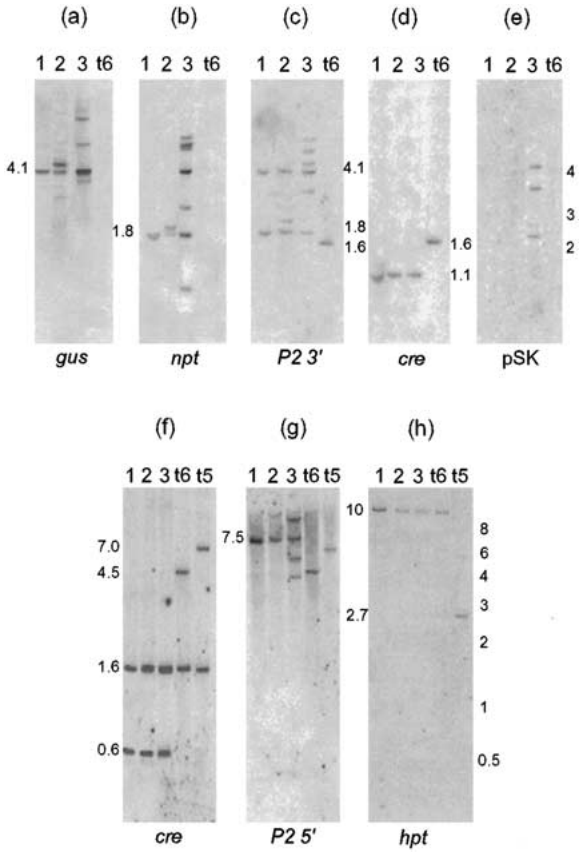

Figure 2. Southern blots of *Eco*RI (a - e) or *Eco*RV (f - h) cleaved DNA from plants regenerated from geneticin-resistant calluses. Lanes 1, 2, 3 are t6-derived Integrant-1, -2, -3, respectively. Each blot was hybridized to the probe indicated below the blot and in Figure 1. Size markers are as indicated.

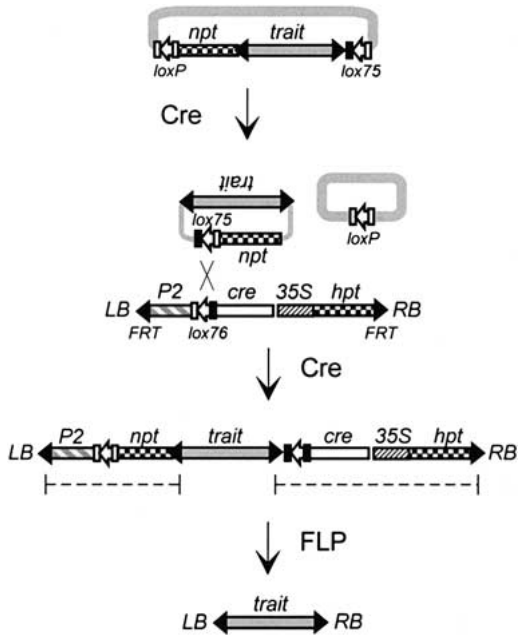

Figure 3. The use of one recombination system to target the delivery of the trait DNA followed by a second recombination system to remove unnecessary gene segments. A simple modification consists of flanking the target site construct and the trait gene in the transformation vector with a pair of inverted recombination sites that are different from those used for site-specific integration. Shown as an example are *FRT* sites (filled arrowheads, as labeled at the target construct) from the yeast FLP-*FRT* recombination system. After Cre-mediated site-specific recombination, the FLP recombinase would be introduced to remove the DNA flanked by the *FRT* sites (indicated by dashed lines).

left border-host DNA junction (Figure 2f). In the integrants, the 1.6 kb band is accompanied by a new 0.6 kb fragment.

DNA external to the *lox* sites was also examined. With *Eco*RI-treated DNA, a 5′ end fragment of *P2 (P2 5′)* or *hpt* detected, respectively, the expected 1.4 kb *P2* fragment or the 1.3 kb *hpt* band in target and integrant lines (data not shown). With *Eco*RV-treated DNA, a fragment >2.8 kb should be detected in t5 and t6 by *cre* or *P2 5*, while in the integrants, only *P2 5′*, but not *cre*, should hybridize to a >5.8 kb band. Figures 2f and 2g show that either *P2 5′* or *cre* detected the same-sized host junctions, 7.0 kb in t5 and 4.5 kb in t6. With the integrants, the host junction detected by *P2 5* increased correspondingly from 4.5 to 7.5 kb (Figure 2g). With the *hpt* probe, a host junction >1.9 kb is expected among target and integrant lines. Indeed, Figure 2h shows a 10 kb band among t6-derived plants and a 2.7 kb fragment in t5.

Each of the bands found in integrant-1 is also present in integrant-2 and integrant-3. However, these two plants have additional bands hybridizing to the *gus, npt, P2 3′* and *P2 5′* probes, which indicates additional copies of pVS55-derived DNA. Since the banding patterns do not suggest tandem DNA repeats at the genomic target, the additional transgene copies are likely to reside elsewhere.

An important consideration of the integration process was the fate of the plasmid backbone that excised from pVS55. A probe consisting of pSK DNA, including portions of the ampicillin resistance gene and the plasmid origin of replication, detected *Eco*RI hybridizing bands in integrant-3 (Figure 2e), which also harbors multiple copies of *gus, npt* and *P2* sequences. However, hybridization was absent in integrant-1, integrant-2, or in t6.

Despite regenerating only a single plant, integrant-1, that has a precise single-copy integration event, this exercise demonstrated the feasibility of biolistic-mediated Cre-*lox* integration in rice. Moreover, the reporter gene was expressed without any signs of gene silencing, as different leaves from this plant stained positive for GUS enzyme activity, with no apparent differences in expression among the leaves (data not shown).

Conclusions

The year-long tissue culture phase could have accounted for the low efficiency of plant regeneration from the integrant calluses. It took four months to generate target line t5 and t6 calluses, four months for the selection of site-specific integration of new DNA into these calluses, and another four months to regenerate shoots from the integrant calluses. In hindsight, the target lines should have been regenerated into plants and then fresh embryogenic calluses reinitiated for the site-specific insertion of new DNA. Of the two false-positive plants regenerated from culture, the t5-escape plant produced seed, and this line remains available for future site-specific integration experiments. Unfortunately, the t6-escape plant was sterile.

Given that only 8% of geniticin-resistant calluses lacked a predicted integrated junction, site-specific integration *via* biolistic delivery can be considered an efficient process. However, half of the transgenic calluses did not show detectable levels of *gus* expression. Of the other half that expressed *gus*, we might expect a large percentage to harbor additional copies of the integrating DNA, as was found in two of the three regenerated plants. Removing the extra transgene copies by genetic segregation might be possible in some instances. Overall, we estimate that an expressed clone which is structurally precise, single-copy and site-specific will likely be recovered in 1 out of 6 isolates.

Future prospects

Site-specific recombination systems can be used for deleting as well as for integrating DNA. A reasonable deduction is that targeted integration can be combined with the subsequent removal of DNA that is no longer needed after transformation (Hohn et al. 2001; Lyznik et al. 1996;Zuo et al. 2001). Figure 3 illustrates how this aim can be achieved with two different recombination systems in a simple modification of the integration strategy shown in this work. The combination of site-specific insertion of a trait gene, followed by the subsequent deletion of unneeded DNA, is an option that can be considered for the engineering of crop plants.

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